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Discovery, Structure and Tentative Functions of a C-Terminal Propeptide of Vacuolar Potato Lipases (Patatins)

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Introduction and Conclusions

Potato tuber patatins amount to 25-40% of potato tuber protein. They are dimers of ca. 90 kDa with lipase/esterase activity as shown by gel filtration followed by activity measurements, whereas the subunits are 40-42 kDa including glycans as demonstrated by non-reducing SDS-PAGE and MALDI-TOF-MS (1). It is well-known that patatins are located in the vacuoles of potato tubers. However, the vacuolar targeting signal has never been identified for this storage and defence protein. Proteome data of potato (*Solanum tuberosum)* tuber juice and of purified potato tuber vacuoles indicated that mature patatins may perhaps lack a ct-propeptide. We have confirmed this by complete mass spectrometric sequencing of a number of patatin variants as well as their N-linked complex-type glycans from the starch-rich cultivar Kuras. For this cultivar full length patatin cDNAs have also been sequenced, as the patatin locus is highly polymorphous. We propose that a six-residues ct-propeptide, -ANKASY-COO^- composes this signal. The crystallographic structure of a recombinant patatin (Rydel et al., 2003, Biochemistry 42, 6696-6708), which included this propeptide thus shows us, for the first time, a putative ligand of the vacuolar sorting receptor and processing enzyme responsible for patatin import and processing.

Protein Sequencing of Mature Patatins

Proteolytic Digestions: MonoQ fractions were precipitated with ice cold ethanol to a final concentration of 60 % over night at - 20 ºC which will precipitate patatins. Pellets were reduced and alkylated prior to digestion in 50 mM NH_4HCO_3, pH 8.0 with sequencing grade modified bovine chymotrypsin, or Lys-C. Samples were digested at 37 ºC at E:S = 100:1 (w/w) for 30 min, and after addition of more protease (1:100) digestions were continued for 1 h and stopped prior to concentration by with vacuum centrifugation. Samples were analyzed by nanoflow capillary high pressure liquid chromatography interfaced directly to an electro spray ionization Q-TOF tandem mass spectrometer (MicroTOFQ, Bruker Daltonics, Bremen, DE), or storage at - 20 °C.

FIGURE 1. **Alignment of translated patatin cDNA sequences using Kuras pat1-k1 as template**

Patatins, purified by Superdex 200 gel filtration and MonoQ anion exchange chromatography, have been subjected to chymotrypsin and Lys-C protease digestions. The complete amino acid sequences were determined for 8 patatins (light blue in Figure 1), except for a few residues in three patatin variants (white in Figure 1). Patatins are targeted to the endoplasmic reticulum (ER) by a typical 23-residues signal peptide which is removed on ER import (yellow in Figure 1). The mature tuber patatin variants are dimers of 40-42 kDa subunits without disulfide bridges, but carries from one to three N-linked glycans (green in Figure 1). Surprisingly, mature patatins purified from potato tubers have so far only been characterized by N-terminal protein sequencing and sequencing of a few peptides (3). The sequences have been translated from cDNA sequences, but never confirmed by complete protein sequencing. Unambiguously, the lists of compiled sequences from monoQ fractions (Table 1 lists peptides assigned to pat2-k3) show that the last six residues of translated Kuras tuber patatins are absent (red in Figure 1) in the mature vacuolar patatins, and thus comprise a ct-propeptide.

Sites of N-linked glycans

Sites of N-Linked Glycans: Mass spectra of glycopeptides were extracted by searching for ions of 204.1 +/- 0.2 for GlcNAc (acetylated glucosamine), 366.1 +/- 0.2 for GlcNAc-Man_1 and 528.2 +/- 0.2 for GlcNAc-Man_2.
Deglycosylation with Glycopeptidase A: Carboxymethylated patatin fractions were prepared and precipitated as described above. The pellet was dissolved in 15 µL 0.1 mM ammonium acetate pH 5 and incubated with 60 mU of glycopeptidase A from almonds for 18 h at 37 °C, dried by vacuum centrifugation, and digested with chymotrypsin as describe above.

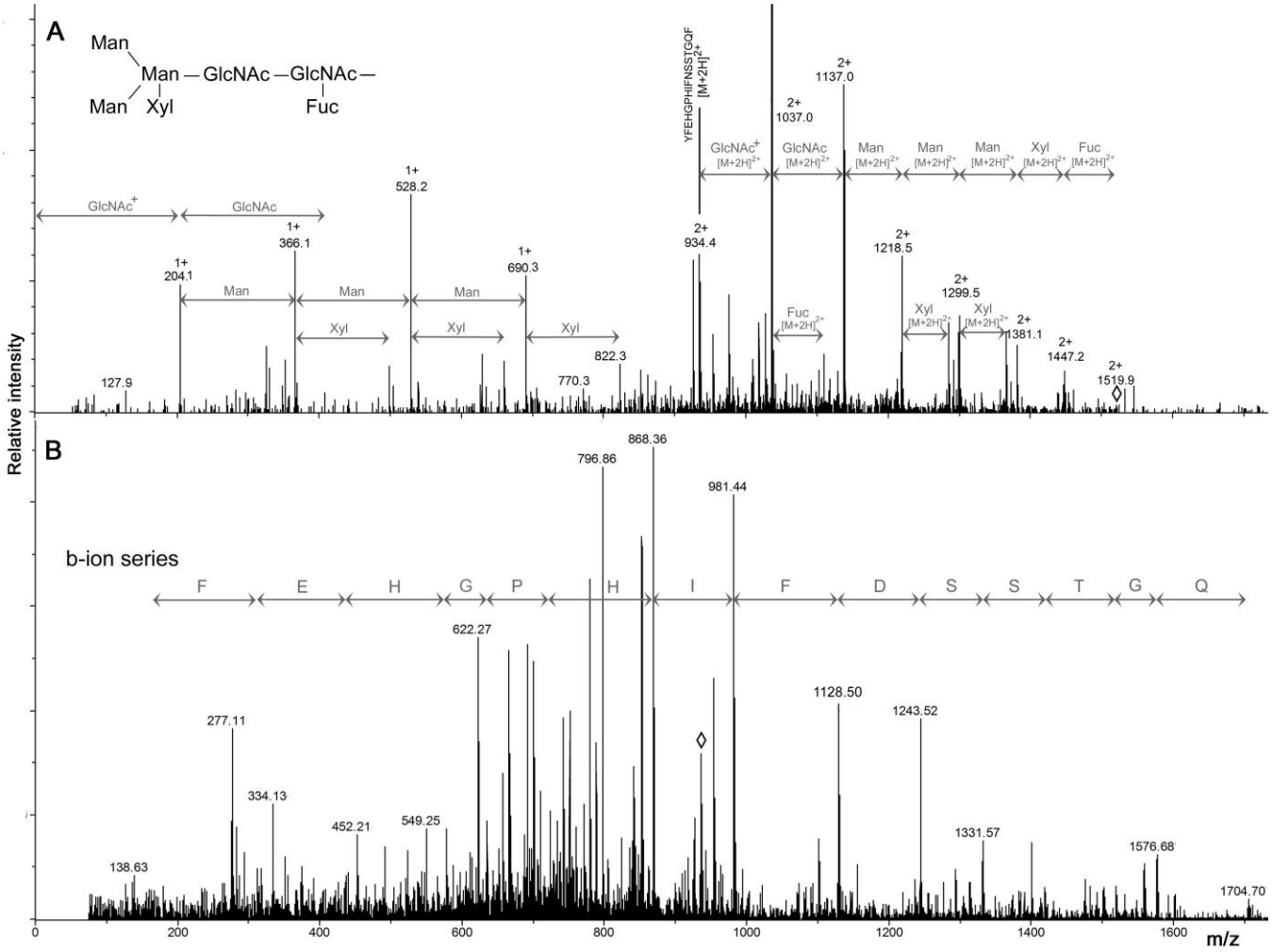

FIGURE 2. **Typical mass spectrometric collision-induced-dissociation fragmentation of a patatin glycopeptide, and the same peptide obtained from enzymatically deglycosylated patatin.**
A, MS/MS spectrum of chymotryptic glycopeptide 106-122 of pat2-k1 and the derived complex-type glycan structure. The double-protonated glycopeptide parent ion is indicated by an open diamond at m/z 1519.9. The double-charged naked peptide is prominent at m/z 934.4. The theoretical average isotopic mass is 1170.4 of the N-linked complex-type glycan. The peptide chain shows little fragmentation, whereas the glycan side chain can be assembled from both the mono- (left) and double-charged ion series (right). *B,* the MS/MS spectrum of the corresponding glycopeptidase A deglycosylated peptide. The double-protonated peptide parent ion is indicated by an open diamond at m/z 934.9, and the peptide sequence derived from the b-ion series is shown.

All potential asparagine-X-threonine/serine glycan acceptor sites (4) in the eight patatins have been verified experimentally (highlighted in green in Figures 1 and 3). All carry a complex glycan, Man_2-Man(Xyl)-GlcNAc-GlcNAc(Fuc)-, typical of plants, and also the most abundant N-linked glycan in potato tuber (5). The MS/MS spectrum of glycopeptide 106-122 of pat2-k1 demonstrates the glycan sequencing (Fig. 2A).
In a separate experiment the S-carboxymethylated patatins were subjected to deglycosylation with glycopeptidase A, and then digested with chymotrypsin. Four different deglycosylated peptides all including position 115 were seen. These deglycosylated peptides were now accessible to normal peptide fragmentation by MS/MS (example in Fig. 2B). The deglycosylated asparagines now appeared as aspartates, the well-known product of enzymatic deglycosylation (6).

Ct-propeptide and vacuolar targeting

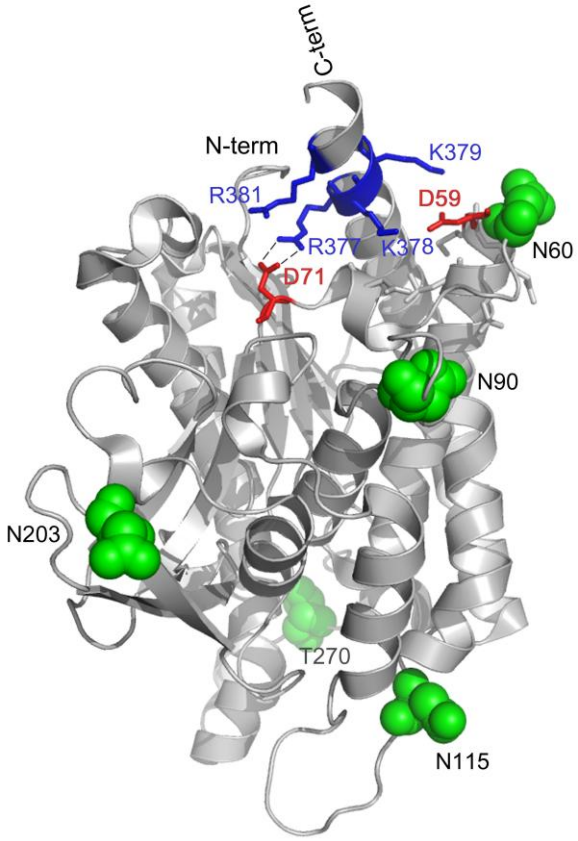

FIGURE 3. **The model of pat-17 molecule A at 2.2 Å resolution (1oxw, www.rcsb.org/pdb)**

The model of pat-17 (7) at 2.2 Å resolution high-lights the locations of glycans and the interactions of the C-terminal residues of mature patatin (Figure 3). The amino acid sequence of pat-17 from *Solanum cardiophyllum* is very similar to patatins pat1-k1 through pat4-k1 from the *Solanum tuberosum* cv Kuras. A recombinant form was expressed in *E. coli* with an N-terminal histidine tag and the ct-propeptide (Figure 1). The structure shows the ct-propeptide sticking out from the remainder of the molecular surface. Therefore, the ct-propeptide including its free α-carboxylate, ANKASY-COO^- is easily accessible to a **vacuolar sorting receptor**. Indeed, the last ct-residues, ASY or ASF, are similar to the ends of many other ct-propeptides of vacuolar storage proteins (reviewed by 8, 9). A homologous cytosolic patatin from *H. brasiliensis*, Hev b 7 (10), ends at position 384 of Figure 1 and has no ct-propeptide-like residues.

Vacuolar proteins are recognized for sorting as discussed, but they are also bound to and cleaved by a vacuolar processing enzyme. We analyzed the interactions of the basic helical C-terminal residues 377-381 (blue side chains) of mature patatins within a 5 Å sphere. Invariant R377 is intimately bound to invariant D71 (red) in a binary mode via short 2.80 and 2.92 Å contacts between the terminal nitrogen atoms of the guanidinium group and the side chain carboxylate oxygens (salt bridge). One of the nitrogens has an additional 2.77 Å hydrogen bond to backbone carbonyl oxygen of methionine28 (residue 4 of mature patatin). The following residues have weak contacts only. The side chain nitrogen of K379 is 3.65 Å away from the carboxylate of D59. Also the contacts of the C-terminal R381 and the N-terminal residues 25LGE27 are weak, because the carboxylate of E27 is pointing away from R381.

We conclude that the C-terminal residues of mature patatins after R377 are loosely bound and, therefore, might easily unfold on binding to a processing enzyme. The processing enzyme is presently unknown in potato tuber vacuoles. As our proteomics data shows the cleavage being exclusively after R381 we propose that it might be an endopeptidase rather than an exopeptidase involved in removal of the six-residues patatin ct-propeptide.

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FIGURE 1. **Alignment of translated patatin cDNA sequences using Kuras pat1-k1 as template**. GenBank accession numbers for pat1-k1 through pat3-k1clones are DQ114415 through DQ 114421. Pat4-k1 has not been cloned and sequenced from cv Kuras; the proteome data scored highest for CAA27571 (translated from gene X03932). Recombinant pat17, rpat-17, from *Solanum cardiophyllum* (AY033231) is shown for comparison, and its structure is shown in Fig. 3.